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Edward M. Postlewhait, Mohammad G. Mustafa, and Nabil M. Elsayed

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Division of Environmental and Occupational Health Sciences, School of Public Health and Div. of Pulmonary Disease, Dept. of Medicine, Center for Health Sciences, U. of CA, Los Angeles, CA 90024

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JOURNAL ARTICLE

AGE-RELATED OZONE EFFECTS ON GLUCOSE UTILIZATION IN RAT LUNGS

Edward M. Postlethwait, Mohammad G. Mustafa*, and Nabil M. Elsayed
Division of Environmental and Occupational Health Sciences
School of Public Health
and
Division of Pulmonary Disease
Department of Medicine
Center for the Health Sciences
University of California
Los Angeles, California 90024

ABSTRACT

We examined the relationship between animal age and pulmonary biochemical effects of O_3 exposure using young to adult rats ranging in age from 24 to 90 days. Glucose uptake and production of pyruvate and lactate, determined in lung tissue slices, were used as indices of O_3-produced metabolic alterations. In control rats, the lung weights increased with animal age until they stabilized between 60 and 90 days of age. The rate of glucose metabolism, expressed per lung, increased with animal age paralleling the increase in lung weights. After exposure to 0.8 ppm (1568 ug/m³) O_3 for 3 days, the lung weights and all three parameters of glucose metabolism increased in exposed rats relative to their age-matched controls. These increases were relatively small for younger rats but much larger for adult rats. Regression of percent differences in these parameters between control and exposed rat lungs against animal age indicates that the response to O_3 was age-related.

INTRODUCTION

The pulmonary biochemical alterations resulting from short-term, low-level exposures to O_3 are well documented (1-3). Typically, exposure of animals to less than 1 ppm (1960 ug/m³) O_3 for a few days results in an increase of pulmonary metabolic activities (1-5). These responses are influenced by such factors as animal species, exposure regimen, and nutritional status (4-16). There is increasing evidence which suggests that the degree of pulmonary response to an oxidant exposure may be influenced by animal age (9, 17-22). However, published reports differ in their assessment of whether young or old animals are more responsive when subjected to oxidant stress. We therefore attempted to study O_3 effects in the lung using young to adult rats, ranging from 24 to 90 days of age, in order to determine the age-dependence of response. We have used several parameters of pulmonary glucose metabolism as the indicators of O_3 effects. Glucose has been shown to be both a major energy substrate and a potential precursor of a variety of metabolites in the lung (23, 24). Alterations in lung glucose uptake and lactate production have been observed with pulmonary edema, and with exposure to hyperoxia, hypoxia, carbon monoxide, and oxidant air pollutants (3, 25-27). These suggest that alterations in glucose metabolism may offer both sensitive and early indicators of lung tissue injury. We have previously shown that glucose metabolism increases in rat lungs due to 0.8 ppm (1568 ug/m³) O_3 exposure, and that peak level increases occur within 3 days of exposure (5). In this study, we have exposed rats of different age groups to 0.8 ppm (1568 ug/m³) O_3 for 3 days, and examined the influence of animal age on pulmonary biochemical effects in terms of alterations in glucose metabolism.

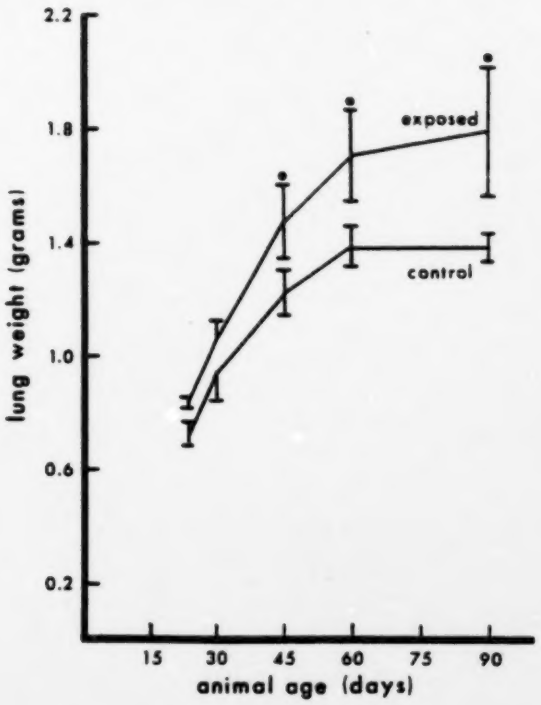


Fig. 1. Effect of 0.8 ppm (1568 ug/m³) O_3 exposure for 3 days on lung weights of rats from different age groups. Each data point represents mean ± SD for 4 rats. The dot (•) refers to statistically significant differences from age-matched controls ($p < 0.05$) by ANOVA.

*To whom all correspondence should be addressed:
School of Public Health
UCLA
Los Angeles, CA 90024

RESULTS

Lung Weight and DNA Content

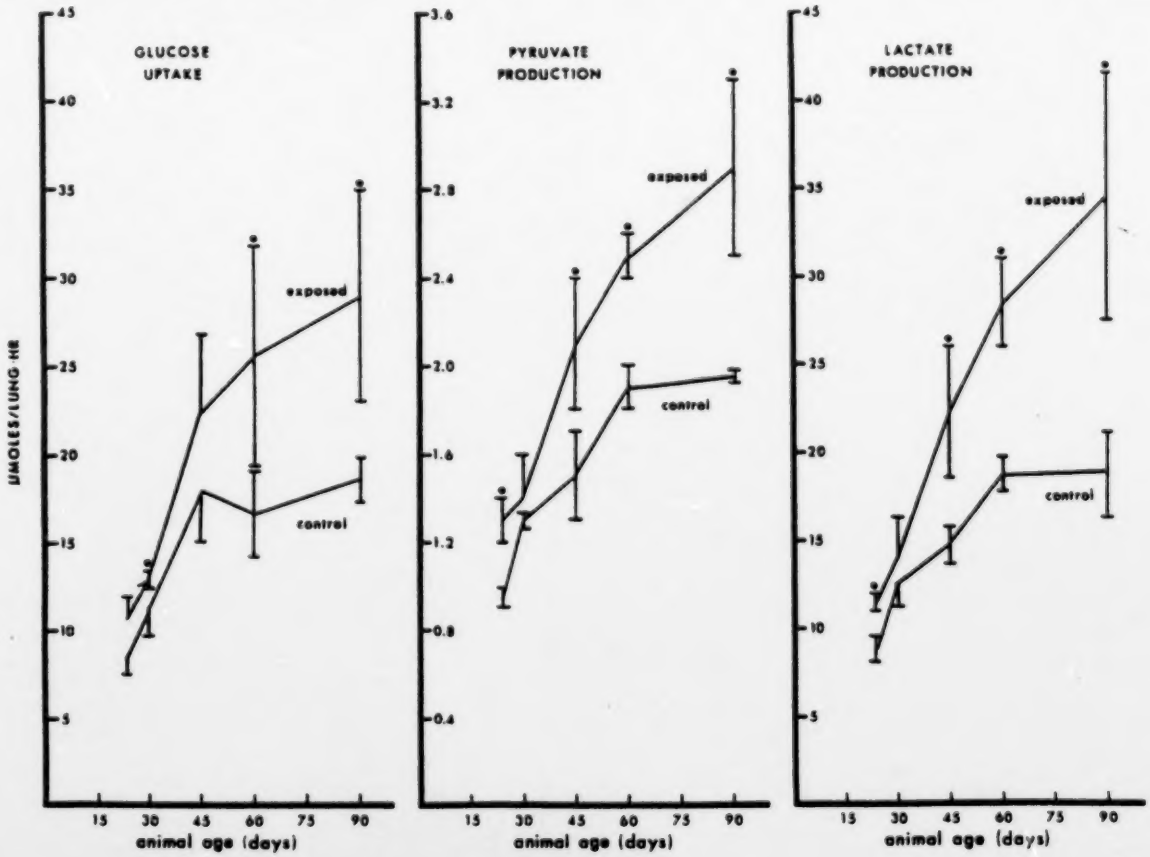
In control rats, body weights increased steadily with age from 112 ± 7 g at 24 days to 447 ± 22 g at 90 days, resulting in approximately a 300% increase. Differences in lung weight and lung DNA content were used to assess the changes in lung tissue mass. Mean lung weights increased with age from 0.72 ± 0.04 at 24 days to 1.38 ± 0.05 g at 60 or 90 days (Fig. 1), which was approximately a 92% increase. Body weights therefore increased at a much greater rate than lung weights. Concomitant with the increase in lung weight, total lung DNA content increased with age from 4.1 ± 0.5 mg at 24 days to 5.6 ± 0.1 mg at 60 or 90 days (Table 1), which was a 37% increase. Therefore, total lung mass increased at a greater rate than did DNA mass.

After exposure to 0.8 ppm (1568 ug/m³) O_3 for 3 days, the body weight gains of various age groups were the same as their age-matched controls. The lung weights of exposed rats increased in all groups compared to their age-matched controls (Fig. 1). The magnitude of these increases was age-dependent: approximately a 15% (nonsignificant) increase for 24-30 day old rats compared to a 30% ($p < 0.05$) increase for 60-90 day old rats. The DNA content increased in all groups relative to their age-matched controls, but the increases were significant for 45 and 60 day old rats only (Table 1). Since both lung weights and DNA content increased with O_3 exposure, the DNA to lung weight ratios did not change relative to age-matched controls, indicating lack of edema.

Histology

Histologically the lungs of all control groups appeared normal. The lungs of exposed rats showed histologic changes, which included thickened septal walls, amorphous alveolar material, and marked thickening in areas around the bronchioalveolar junction. However, there was no obvious accumulation of fluid in either the perivascular or

Fig. 2. Effect of 0.8 ppm (1568 ug/m³) O_3 exposure for 3 days on glucose uptake and production of pyruvate and lactate in the lungs of rats from different age groups. Each data point represents mean ± SD for 4 rats. The dot (o) refers to statistically significant differences from age-matched controls ($p < 0.05$) by ANOVA.



peribronchial spaces, and the alveolar spaces appeared dry.

Glucose Metabolism

The rates of glucose utilization are shown in Fig. 2. In control rats, glucose consumption, expressed per lung (umoles/lung.hr), increased with age from 8.4 ± 0.8 at 24 days to 18.6 ± 1.3 at 90 days. the production of both pyruvate and lactate increased with age. When the results for all three parameters were expressed per gram of lung tissue (umoles/g.hr), there were no significant differences in these rates among the age groups. After O_3 exposure, the per lung rate of glucose uptake increased in all exposed animal groups relative to their controls (Fig. 2). The relative differences in the rates between control and exposed animals increased with age, e.g. 25% (nonsignificant) for 24 day old rats compared to 55% ($p < 0.05$) for 90 day old rats. Production of pyruvate and lactate was affected by O_3 exposure in a similar manner, with the largest increases over age-matched controls occurring in the oldest animal group. Regression of the percent differences between the control and exposed rat lungs for all three parameters against animal age gave a line with a positive slope (Fig. 3), showing an increase in relative response with increasing animal age. When the metabolic rates in lung tissue from exposed rats were expressed per gram, they showed slight elevations (10-20%) relative to age-matched controls, but the differences were not statistically significant.

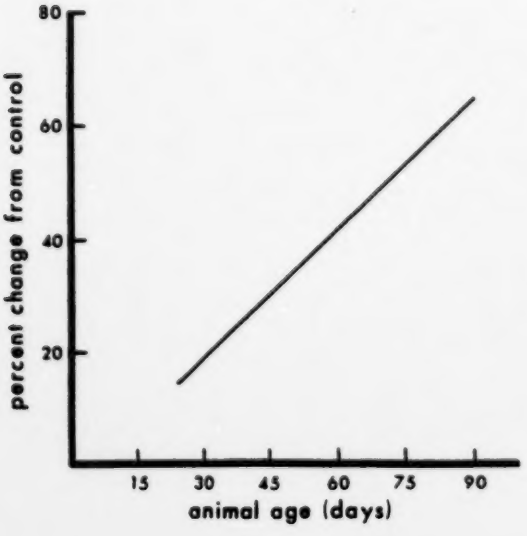


Fig. 3. A regression of percent differences between exposed and age-matched control rat lungs for glucose uptake and production of pyruvate and lactate against animal age ($r^2 = 0.7$).

DISCUSSION

The results of this study demonstrate that with growth, rat body weights increased at a faster rate than lung weights. This means that with age the lungs must serve increasingly larger bodies, and this may have relevance to the age-related response of animals to inhaled O_3. With age, the lung mass increased at a faster rate than did DNA mass, suggesting that lung weight increased more as a result of cellular enlargement and accumulation of non-cellular matrix rather than of cellular multiplication (28-30). The results of this study further demonstrate that glucose uptake and production of pyruvate and lactate in lungs increased with animal age. However, the age-related metabolic increases were observed when the rates were expressed per whole lung but not per gram of lung tissue. The whole organ metabolic rates werre therefore dependent upon the lung mass.

After O_3 exposure, the lung weights increased in all age groups, but the larger increases occurred in 60-90 day old rats compared to 24-30 day old rats. The DNA content increased in exposed rat lungs but less appreciably. It is possible that an increase in DNA content due to multiplication of surviving cells and influx of inflammatory cells was in part balanced by a loss of injured or dead cells. The rates of lung glucose metabolism increased in exposed rats relative to their age-matched controls. The increases were appreciable and statistically significant when expressed per whole lung but small and nonsignificant when expressed per gram of lung tissue. The O_3-induced metabolic increases were therefore primarily dependent upon the lung mass. However, the small (nonsignificant) increases per gram of lung tissue might be accounted for by a change in lung cell population. Of particular note is that the increases in metabolic rates after O_3 exposure were larger for older animals. A plot of the regressed percent differences against age showed an increased response with increasing age.

An augmentation of lung metabolism, e.g. glucose utilization used in this study, observed after O_3 or NO_2 exposure is thought to represent a response of lung tissue to injury caused by oxidative and free radical-mediated reactions (1-3). Both O_3 and NO_2 exposures can cause lung injury producing similar biochemical and morphological alterations, although the extent of these alterations may differ quantitatively (31, 32). Most typically, ciliated epithelial cells of the terminal bronchioles and type I epithelial cells of the proximal

Table 1. Effect of 0.8 ppm (1568 ug/m³) O_3 exposure for 3 days on DNA content in rat lung

Animal Age	Values (mg/lung)[a] Control	Exposed	% Increase
24	4.1 ± 0.5	4.6 ± 0.8	12
30	4.8 ± 0.7	5.4 ± 0.4	17
45	5.6 ± 0.4	6.7 ± 0.9	20[b]
60	5.6 ± 0.1	6.7 ± 0.3	20[b]
90	5.6 ± 0.2	6.3 ± 0.4	13

[a] Data represent mean ± SD; n = 4.

[b] Significant to $p < 0.05$ by ANOVA.

alveoli are damaged, followed by a proliferation of Clara cells in the terminal bronchioles and type II epithelial cells in the alveoli. The proliferation of these cells and increased lung metabolism follow a similar time-course during O_3 or NO_2 exposure (1, 3-8). Cabral-Anderson *et al.* (18) and Evans *et al.* (19) exposed rats of different age groups to NO_2, and observed that older rats received much greater lung injury particularly in the alveolar region. Because of the similarity in lung injury caused by O_3 and NO_2, it is likely that a similar pattern of response occurred with O_3 exposure used in the present study. Other adverse conditions, specifically pulmonary edema, have been associated with alterations of lung glucose metabolism (27, 33). However, light microscopic examinations of histologic sections did not detect any edema in exposed rat lungs. In addition, the intra-group DNA to lung weight ratios were not statistically different between control and exposed rats, suggesting that the relative increases in DNA and lung weight due to O_3 exposure occurred to the same extent. Thus, increased lung weights and glucose metabolism might be accounted for by such cell population changes as proliferation of metabolically active cells and influx of inflammatory cells.

The differences in age-related response observed in this study may have been generated by a number of different situations. As suggested for NO_2 exposure (18, 29), the O_3 concentration used in our study may have caused a greater lung injury in older rats than in younger rats, and hence the greater response evoked in older animals. Alternatively, the O_3 exposure may have generated a similar degree of lung injury in rats of all age groups, but the response was greater in older animals. Another possibility is that the time-course of injury and response during O_3 exposure is different for young versus old rats. We used the 3-day exposure period as the peak response time because we previously observed this repeatedly in 60-70 day old rats (4, 5, 7). If it so happens that younger rats have a slower response time to O_3 injury than older rats even though the ultimate response may or may not amount to be the same for all age groups, our current interpretations should nonetheless hold true. However, presently we do not have any data to suggest whether the time-course of injury and response is indeed different for young versus old animals.

It may be concluded that O_3 exposure causes an alteration of lung glucose uptake and production of pyruvate and lactate more in adult rats than in young rats. Animal age is therefore an important factor to consider when selecting a sensitive animal model for studying the pulmonary effects of inhaled oxidants.

EXPERIMENTAL PROCEDURES

Animals and Ozone Exposure

Specific pathogen free, male Sprague-Dawley rats ages 24, 30, 45, 60 and 90 days were obtained from Hilltop Laboratories (Chatsworth, Calif.). Rats from each age group were exposed to 0.8 ppm (1568 ug/m^3) O_3 continuously for 3 days in a stainless steel inhalation chamber (Young and Bertke, Cincinnati, Ohio). Ozone was generated by passing 100% O_2 (0.5 l/min) through an ozonizer (Sander, Peine Am Osterberg, W. Germany) and was mixed with filtered air at the chamber inlet to give the desired concentration. The chamber O_3 concentration was continuously monitored using a Dasibi O_3 monitor (model 1003-PC, Environmental Corp., Glendale, Calif.). Control animals were maintained in a similar chamber supplied with filtered room air. The air flow in both chambers was kept at 20 volume changes per hour. All animals were allowed free access to food and water during the experimental period.

Tissue Preparation and Assays

After O_3 exposure, the rats were weighed, and then anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and sacrificed by exsanguination. The lungs were quickly removed, individual lobes dissected free at the hila, blotted once on cotton gauze, and weighed. A portion of lung tissue was fixed in Bouin's fixative for histologic examination, and another portion set aside for DNA determination.

For assay of glucose metabolism, 100 mg of 1 mm thick tissue slices, prepared with a McIlwain tissue chopper (Brinkman Instruments, New York), were placed in a 25 ml Erlenmeyer flask containing 2 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) and 8.33 mM glucose. The flasks were then sealed with rubber stoppers, gassed with 95% O_2/5% CO_2 and incubated in a rotary shaker for 90 minutes at 37°C. The tissue samples were incubated in triplicate with accompanying buffer blanks. At the end of incubation, a 1 ml aliquot was transferred to a tube, mixed with 3 ml of 6% perchloric acid, centrifuged at 3,000 x g for 10 minutes, and the supernatant collected for analysis. Glucose uptake was determined from the difference in glucose concentrations between flasks with and without tissue by a glucose oxidase method (Sigma Chemical Co., St. Louis, Mo.). Pyruvate and lactate were determined enzymatically (Sigma Chemical Co.), and the production calculated from the difference between flasks with and without tissue. Lung tissue DNA content was determined by the method of Schneider (34). Lung samples fixed for histology were sliced into 5 um sections and stained with hematoxylin and eosin.

Data Presentation

The results, expressed per whole organ (lung), are presented as mean ± one standard deviation (SD).

Statistical significance was determined by two-way analysis of variance with multiple t comparisons, and $p < 0.05$ was considered significant (35). In addition, the percent differences between the exposed and control lungs for all three metabolic parameters were regressed against animal age in order to demonstrate the relationship between animal age and response to O_3 insult.

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DISCLAIMER

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REFERENCES

1. Cross, C.E., De Lucia, A.J., Reddy, A.K., Hussain, M.Z., Chow, C.K., and Mustafa, M.G.: Ozone interaction with lung tissue: Biochemical approaches. Am. J. Med., 60: 929-935, 1976.

2. Coffin, D.L. and Stokinger, H.E.: Biological effects of air pollutants, edited by Stern, A.C., Academic Press, New York, 1977, Vol. 2, pp. 231-361.

3. Mustafa, M.G. and Tierney, D.F.: State of the art — biochemical and metabolic changes in the lung with oxygen, ozone, and nitrogen dioxide toxicity. Am. Rev. Respir. Dis., 118: 1061-1090, 1978.

4. Mustafa, M.G. and Lee, S.D.: Pulmonary biochemical alterations resulting from ozone exposure. Ann. Occup. Hyg., 19: 17-26, 1976.

5. Mustafa, M.G., Hacker, A.D., Ospital, J.J., Hussain, M.Z., and Lee, S.D.: Biochemical effects of environmental oxidant pollutants in animal lungs, in Biochemical Effects of Environmental Pollutants, edited by Lee, S.D., Ann Arbor Science, Michigan, 1977, pp. 59-96.

6. Dungworth, D.L., Castleman, W.L., Chow, C.K., Mellick, P.W., Mustafa, M.G., Tarkington, B.K., and Tyler, W.S.: Effect of ambient levels of ozone on monkeys. Fed. Proc., 34: 1670-1674, 1975.

7. Schwartz, L.W., Dungworth, D.L., Mustafa, M.G., Tarkington, B.K., and Tyler, W.S.: Pulmonary responses of rats to ambient levels of ozone. Lab. Invest., 34: 565-578, 1976.

8. Evans, M.J., Stephens, R.J., and Freeman, G.: Renewal of pulmonary epithelium following oxidant injury, in Lung Cells in Disease, edited by Bouhuys, A., Elsevier, New Y ork, 1976, pp. 165-178.

9. Stokinger, H.E.: Evaluation of the hazards of ozone and oxides of nitrogen. Arch. Indust. Health, 15: 181-190, 1957.

10. Goldstein, B.D., Buckley, R.D., Cardenas, R., and Balchum, O.J.: Ozone and vitamin E. Science, 169: 605-606, 1970.

11. Chow, C.K. and Tappel, A.L.: An enzymatic protective mechanism against lipid peroxidation damage to lungs of ozone-exposed rats. Lipids, 7: 518-524, 1972.

12. Roehm, J.N., Hadley, J.G., and Menzel, D.B.: Antioxidant vs. lung disease. Arch. Intern. Med., 124: 88-93, 1971.

13. Fletcher, B.L. and Tappel, A.L.: Protective effects of dietary alpha-tocopherol in rats exposed to toxic levels of ozone and nitrogen dioxide. Environ. Res., 6: 165-175, 1973.

14. Mustafa, M.G.: Influence of dietary vitamin E on lung cellular sensitivity to ozone. Nutr. Reports Int'l., 11: 473-476, 1975.

15. Mustafa, M.G., Elsayed, N.M., Quinn, C.L., Postlethwait, E.M., Gardner, D.E., and Graham, J.A.: Comparison of pulmonary biochemical effects of low-level ozone exposure on mice and rats. J. Toxicol. Environ. Health, 9: 857-865, 1982.

16. Elsayed, N.M., Hacker, A.D., Kuehn, K., Mustafa, M.G., and Schrauzer, G.N.: Dietary antioxidants and biochemical response to oxidant inhalation. II. Influence of dietary selenium on the biochemical effects of ozone exposure in mouse lung. Toxicol. Appl. Pharmacol., 71: 398-406, 1983.

17. Bils, R.F.: Ultrastructural alterations of alveolar tissue of mice. III. Ozone. Arch. Environ. Health, 20: 468-480, 1970.

18. Cabral-Anderson, L.J., Evans, M.J., and Freeman, G.: Effects of NO_2 on the lungs of aging rats. I. Morphology. Exp. Mol. Pathol., 27: 353-365, 1977.

19. Evans, M.J., Cabral-Anderson, L.J., and Freeman, G.: Effects of NO_2 on the lungs of aging rats. II. Cell Proliferation. Exp. Mol. Pathol., 27: 366-376, 1977.

20. Tierney, D.F., Ayers, L., and Kasuyama, R.S.: Altered sensitivity to oxygen toxicity. Am. Rev. Respir. Dis. 115 (Supplement): 59-65, 1977.

21. Yam, J., Frank, L., and Roberts, R.J.: Oxygen toxicity: comparison of lung biochemical responses in neonatal and adult rats. Pediat. Res., 12: 115-119, 1978.

22. Elsayed, N.M., Mustafa, M.G., and Postlethwait, E.M.: Age-dependent pulmonary response of rats to ozone exposure. J. Toxicol. Environ. Health, 9: 835-848, 1982.

23. Yeager, H. and Massaro, D.: Glucose metabolism and glycoprotein synthesis by lung slices. J. Appl. Physiol., 32: 477-482, 1972.

24. Tierney, D.F. and Levy, S.E.: Glucose metabolism, in Lung Biology in Health and Disease, Vol. 2, The Biochemical Basis of Pulmonary Function, edited by Crystal, R.G., Marcel Dekker, New York, 1976, pp. 105-125.

25. Bassett, D.J.P. and Fisher, A.B.: Metabolic response to carbon monoxide by isolated rat lungs. Am. J. Physiol., 230: 658-663, 1976.

26. Bassett, D.J.P. and Fisher, A.B.: Glucose metabolism in rat lung during exposure to hyperbaric O_2. J. Appl. Physiol., 45: 943-949, 1979.

27. Young, S.L., O'Neil, J.J., Kasuyama, R.S., and Tierney, D.F.: Glucose utilization by edematous rat lungs. Lung, 157: 165-177, 1980.

28. Enesco, M. and Leblond, C.P.: Increase in cell number as a factor in the growth of the organs and tissues of the young male rat. J. Embryol. Exp. Morphol., 10: 530-562, 1962.

29. Winick, M. and Noble, A.: Quantitative changes in DNA, RNA, and protein during prenatal and postnatal growth in rat. Dev. Biol., 12: 451-466, 1965.

30. Kauffman, S.L.: Cell proliferation in the mammalian lung. Int. Rev. Exp. Pathol., 22: 131-191, 1980.

31. Freeman, G., Juhos, L.T., Furiosi, N.J., Mussenden, R., Stephens, R.J., and Evans, M.J.: Pathology of pulmonary disease from exposure to interdependent ambient gases (nitrogen dioxide and ozone). Arch. Environ. Health, 29: 203-210, 1974.

32. Mustafa, M.G., Elsayed, N.M., von Dohlen, F.M., Hassett, C.M., Postlethwait, E.M., Quinn, C.L., Graham, J.A., and Gardner, D.E.: A comparison of biochemical effects of nitrogen dioxide, ozone, and their combination in mouse lung. Toxicol. Appl. Pharmacol., 72: 82-90, 1984.

33. Postlethwait, E.M. and Young, S.L.: Alteration of rat lung adenine nucleotide content after pulmonary edema. Lung, 158: 157-164, 1980.

34. Schneider, W.C.: Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., 161: 292-303, 1945.

35. Dunn. O.S. and Clark, V.A.: Applied Statistics: Analysis of Variance and Regression, John Wiley, New York, 1974, pp. 48-249.